

## RESEARCH ARTICLE

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# SspA up-regulates gene expression of the LEE pathogenicity island by decreasing H-NS levels in enterohemorrhagic *Escherichia coli*

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## Abstract

**Background:** Enterohemorrhagic *Escherichia coli* (EHEC) colonizes the intestinal epithelium and causes attaching and effacing (A/E) lesions. Expression of virulence genes, particularly those from the locus of the enterocyte effacement (LEE) pathogenicity island is required for the formation of a type three secretion system, which induces A/E lesion formation. Like other horizontally acquired genetic elements, expression of the LEE is negatively regulated by H-NS. In the non-pathogenic *Escherichia coli* K-12 strain the stringent starvation protein A (SspA) inhibits accumulation of H-NS, and thereby allows de-repression of the H-NS regulon during the stationary phase of growth. However, the effect of SspA on the expression of H-NS-controlled virulence genes in EHEC is unknown.

**Results:** Here we assess the effect of SspA on virulence gene expression in EHEC. We show that transcription of virulence genes including those of the LEE is decreased in an *sspA* mutant, rendering the mutant strain defective in forming A/E lesions. A surface exposed pocket of SspA is functionally important for the regulation of the LEE and for the A/E phenotype. Increased expression of *ler* alleviates LEE expression in an *sspA* mutant, suggesting that the level of Ler in the mutant is insufficient to counteract H-NS-mediated repression. We demonstrate that the H-NS level is two-fold higher in an *sspA* mutant compared to wild type, and that the defects of the *sspA* mutant are suppressed by an *hns* null mutation, indicating that *hns* is epistatic to *sspA* in regulating H-NS repressed virulence genes.

**Conclusions:** SspA positively regulates the expression of EHEC virulence factors by restricting the intracellular level of H-NS. Since SspA is conserved in many bacterial pathogens containing horizontally acquired pathogenicity islands controlled by H-NS, our study suggests a common mechanism whereby SspA potentially regulates the expression of virulence genes in these pathogens.

**Keywords:** Enterohemorrhagic *Escherichia coli*, LEE, SspA, H-NS

## Background

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is an emerging food- and waterborne- enteric pathogen causing diarrhea, hemorrhagic colitis and the potentially fatal complication hemolytic uremic syndrome in humans [1,2]. EHEC colonization of enterocytes of the large bowel is characterized by an intestinal attaching and effacing (A/E) histopathology, which is manifested by a localized degeneration of brush border microvilli and an intimate

attachment of bacteria to actin-rich pedestal-like structures formed on the apical membrane directly beneath adherent bacteria [3]. The A/E lesion is due to the activity of a type III secretion system (T3SS) mainly encoded by the 35–45 kb locus of enterocyte effacement pathogenicity island (hereafter named LEE), which is conserved in some EHEC isolates and other A/E pathogens such as enteropathogenic *Escherichia coli* (EPEC), atypical EPEC, rabbit EPEC, *Escherichia albertii* and *Citrobacter rodentium* [4–7]. The LEE pathogenicity island comprises at least 41 genes that mainly are located in five major operons (*LEE1-5*). The LEE encodes a TTSS, translocator proteins, secreted effectors, regulators, an intimin (adhesin) and a translocated intimin receptor. The LEE-encoded

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regulators Ler, Mpc, GrlR and GrlA are required for proper transcriptional regulation of both LEE- and non-LEE-encoded virulence genes in response to environmental cues [8–12].

The LEE was acquired by horizontal gene transfer [13] and is regulated by both generic *E. coli*- and pathogen-specific transcription factors. Consequently, the regulation of the LEE reflects characteristics of such genetic elements (For review see [11,14]). Silencing of xenogeneic DNA in bacterial pathogens under conditions unfavorable for infection is important to ensure bacterial fitness [15]. H-NS, which is an abundant pleiotropic negative modulator of genes involved in environmental adaptation and virulence [16–20], is a major silencing factor of horizontally acquired genes [21,22]. H-NS silences genes in the H-NS regulon by various mechanisms. Binding of H-NS to regulatory regions of these genes prevents RNA polymerase from accessing and escaping from promoter DNA, which represents two different mechanisms used by H-NS to silence gene expression (see [23–25] and references therein). H-NS is also a major transcriptional modulator of the LEE pathogenicity island, where it negatively affects the expression of *LEE1-5*, *map* and *grlRA* [26–31]. Further, H-NS binds to regulatory sequences upstream of virulence-associated genes located outside of the LEE including those encoding the long polar fimbriae (*lpf*) required for intestine cell adherence and enterohemolysin (*ehx*) [32,33].

The expression of EHEC virulence genes including those encoded by the LEE is derepressed from the H-NS-mediated transcriptional silencing under physiological conditions that EHEC encounters during infection. Also, LEE expression is growth phase-dependent with maximum expression in early stationary phase [34]. H-NS-mediated silencing of transcription is overcome by the action of DNA-binding H-NS paralogues such as the *LEE1*-encoded global transcriptional regulator Ler (For review see [35]). Ler promotes the expression of many H-NS-repressed virulence genes including those of *LEE1-5*, *grlRA* and non-LEE-encoded virulence genes such as *lpf* and the virulence plasmid pO157-encoded mucinase *stcE* [26,28,31,36–39]. Thus, Ler antagonizes H-NS in the regulation of many virulence genes, which belong to both the H-NS and Ler (H-NS/Ler) regulons.

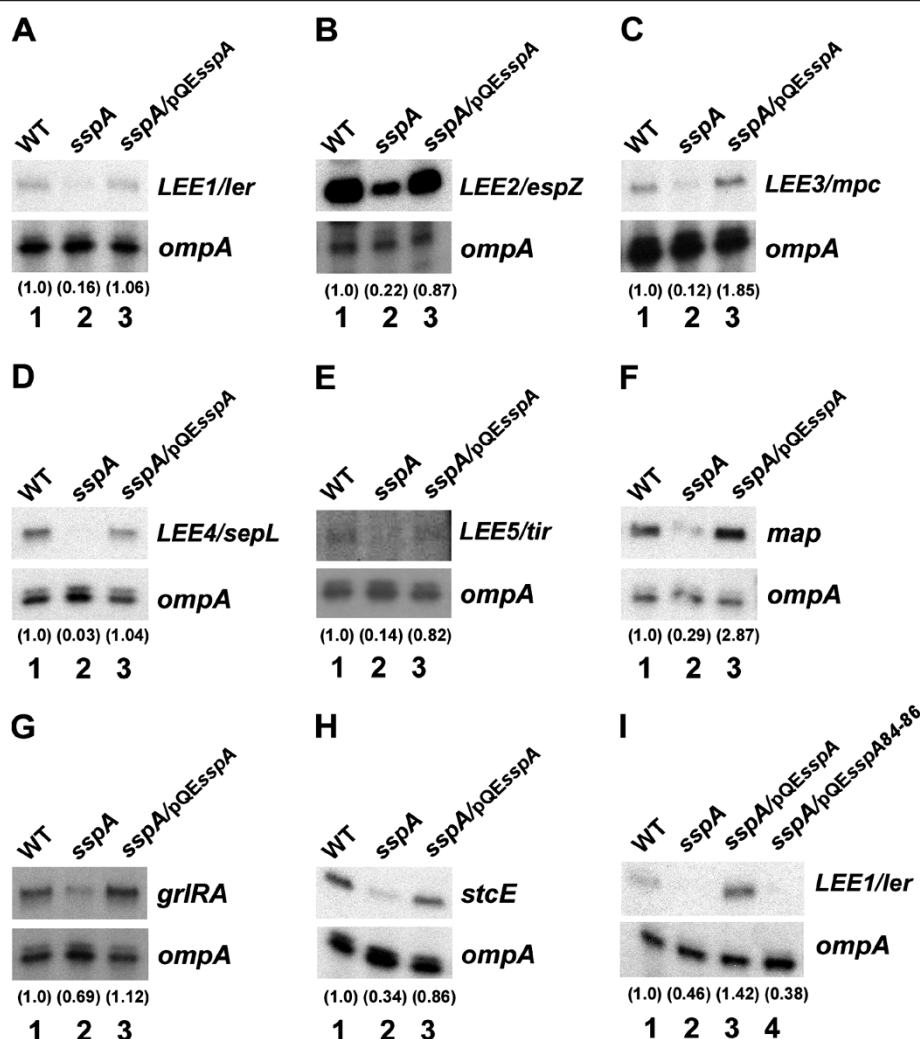
The *E. coli* stringent starvation protein A (SspA) is a RNA polymerase-associated protein [40] that is required for transcriptional activation of bacteriophage P1 late genes and is important for survival of *E. coli* K-12 during nutrient depletion and prolonged stationary phase [41–43]. Importantly, SspA down-regulates the cellular H-NS level during stationary phase, and thereby derepresses the H-NS regulon including genes for stationary phase induced acid tolerance in *E. coli* K-12 [44]. A conserved surface-exposed pocket of SspA is important for its

activity as a triple alanine substitution P84A/H85A/P86A in surface pocket residues abolishes SspA activity [45]. SspA is highly conserved among Gram-negative pathogens [44], which suggests a role of SspA in bacterial pathogenesis. Indeed, SspA orthologs affect the virulence of *Yersinia enterocolitica*, *Neisseria gonorrhoeae*, *Vibrio cholerae*, *Francisella tularensis* and *Francisella novicida* [46–51]. Since *E. coli* K-12 SspA is conserved in EHEC where H-NS negatively modulates virulence gene expression, we asked the question of whether SspA-mediated regulation of H-NS affects EHEC virulence gene expression. Here we study the effect of SspA on the expression of LEE- and non-LEE-encoded virulence genes and its effect on H-NS accumulation in EHEC. Our results show that in an *sspA* mutant elevated levels of H-NS repress the expression of virulence genes encoding the T3SS system rendering the cells incapable of forming A/E lesions. Thus, our data indicate that SspA positively regulates stationary phase-induced expression of H-NS-controlled virulence genes in EHEC by restricting the H-NS level.

## Results and discussion

### SspA positively affects transcription of EHEC virulence genes

To evaluate the effect of *sspA* on virulence gene expression in EHEC during the stationary phase we constructed an in-frame deletion of *sspA* in the *E. coli* O157:H7 strain EDL933 ATCC 700927 [52] and measured transcription of LEE- (*LEE1-5*, *grlRA* and *map*) and non-LEE-encoded (*stcE* encoded by pO157) genes (Figure 1). Wild type and *sspA* mutant strains were grown in LB medium to stationary phase with similar growth rates (data not shown). Total RNA was isolated and transcript abundance was measured by primer extension analyses using labeled DNA oligos specific to each transcript of interest and *ompA*, which served as internal control for total RNA levels. Results revealed that transcript levels of *LEE1-5*, *grlRA*, *map* and *stcE* were reduced by up to 8-fold in the *sspA* mutant compared to wild type (Figure 1A–H, lanes 1 and 2). The expression of these genes was restored when the *sspA* mutant was supplied with wild type *sspA in trans* from pQE*sspA* [43] (Figure 1A–H, lane 3). However, the expression of *ler* and other virulence genes tested (*grlRA*, *espZ*, *sepL* and *stcE*) remained repressed when the *sspA* mutant strain was supplied with mutant *sspA* from pQE*sspA*84–86 [45], which expresses SspA containing the tri-ple alanine substitution in the surface-exposed pocket (Figure 1I and data not shown). These results indicate that SspA positively affects stationary phase-induced expression of both LEE- and non-LEE-encoded virulence genes in EHEC. Moreover, the mode of action of SspA is likely similar in *E. coli* K-12 and EHEC as the surface-exposed



**Figure 1** *SspA* positively affects LEE expression in stationary phase cells. Primer extension analyses on total RNA extracted from wild type EHEC EDL933 (lane 1), the *sspA* mutant (lane 2) and the *sspA* mutant complemented with wild type *sspA* (lane 3) or mutant *sspA84-86* (lane 4) as indicated, grown in LB at 37°C to stationary phase ( $OD_{600} \sim 3.0$ ). The Labeled DNA oligos specific to the transcripts of *LEE1/ler* (A and I), *LEE2/espZ* (B), *LEE3/mpc* (C), *LEE4/sepL* (D), *LEE5/tir* (E), *map* (F), *grlRA* (G) and *stcE* (H) were used. The *ompA* transcripts, detected with a labeled *ompA*-specific DNA oligo, served as internal control for the primer extension reaction. Wild type and mutant *SspA* were expressed from pQE*sspA* and pQE*sspA84-86* respectively in the absence of induction at similar levels. The transcripts *LEE1-5*, *map*, *grlRA*, *stcE* and the control transcript *ompA* are indicated. The relative transcript levels of target genes normalized to that of *ompA* are indicated by the numbers in parenthesis.

pocket of *SspA* also is required for *SspA* to affect the expression of EHEC virulence genes.

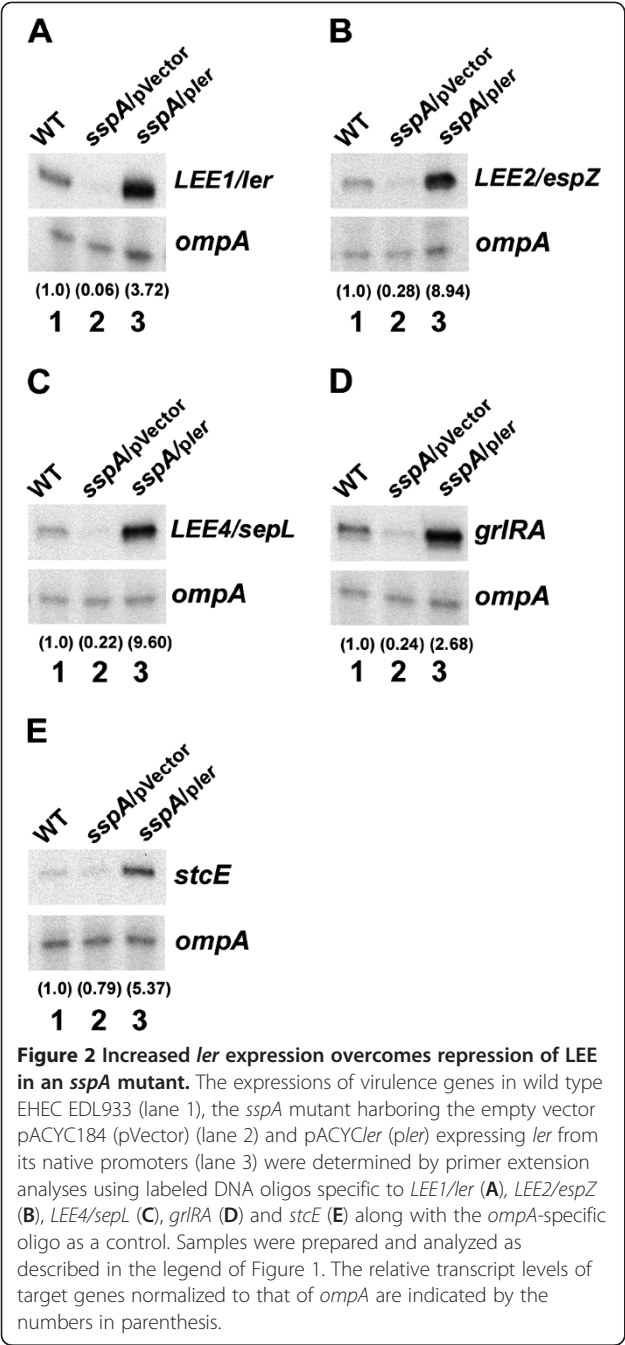
#### Increased expression of *ler* enhances expression of virulence genes in the *sspA* mutant

A decreased expression of *ler* in the *sspA* mutant (Figure 1A) could account for the apparent transcriptional repression of *LEE2-5*, *grlRA*, *map* and *stcE* (Figure 1B-H) because *Ler* positively controls those genes. Thus, we examined whether supplying *ler in trans* from the plasmid pACYC*ler* would alleviate the expression of *Ler*-regulated genes in an *sspA* mutant (Figure 2). Our results showed that transcript levels of *LEE1*, *LEE2*, *LEE4*, *grlRA* and

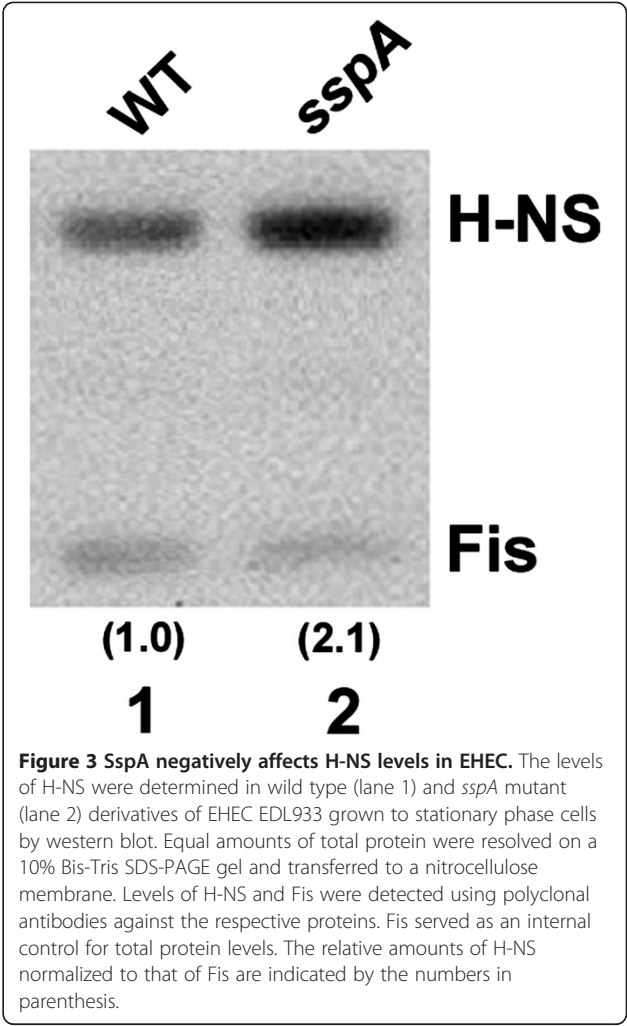
*stcE* were all increased in the *sspA* mutant harboring pACYC*ler* and exceeded that in wild type with up to about 9-fold (Figure 2A-E, compare lanes 1 and 3). These results are consistent with the explanation that a reduced expression of *ler* in the *sspA* mutant leads to an insufficient amount of *Ler* to antagonize H-NS-mediated repression of those virulence genes.

#### *SspA* activates virulence gene expression by reducing the H-NS level

Reduced virulence gene expression during the stationary phase could also be due to an increased level of H-NS in the EHEC *sspA* mutant as observed for H-NS-regulated



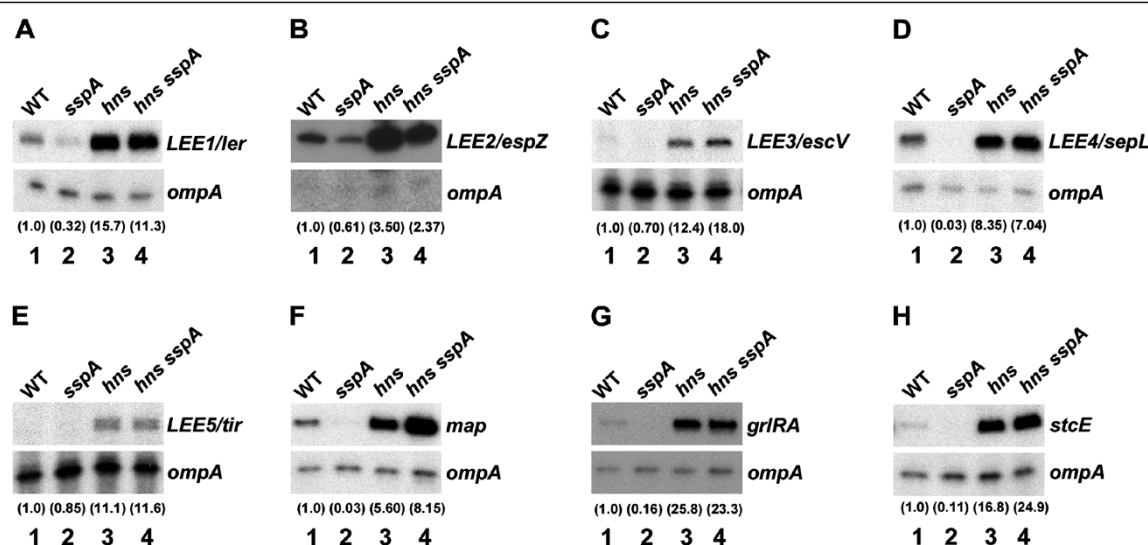
genes in the *E. coli* K-12 *sspA* mutant [44]. We measured the levels of H-NS in stationary phase cells of wild type and *sspA* mutant EHEC strains by western analysis (Figure 3). Indeed, the H-NS level was two-fold higher in the *sspA* mutant than in the wild type, whereas the level of Fis as a control was not increased in the mutant compared to wild type. These results indicate that SspA activates the expression of EHEC virulence genes by decreasing accumulation of H-NS. Notably, such relative small change in H-NS levels was previously demonstrated to drastically affect the expression of the H-NS regulon



involved in stationary phase-induced acid tolerance of *E. coli* K-12 [44].

Genetic analysis further indicated that *hns* mainly is epistatic to *sspA* in regulating H-NS-repressed virulence genes in EHEC (Figure 4). We deleted *hns* in EHEC wild type and *sspA* mutant strains as described in *Methods*. The EHEC *hns* mutant derivatives had a mucoid phenotype and a longer generation time ( $g_{\text{WT}} \sim 27$ ,  $g_{\text{hns}} \sim 36$  min and  $g_{\text{hns,sspA}} \sim 45$  min). Therefore, at least two independent clones of each *hns* mutant derivative were used in each experiment to ensure reproducible results. The expression of *LEE1-5*, *grlRA*, *map* and *stcE* was between 4 and 26-fold higher in an isogenic *hns* null mutant than in wild type (Figure 4A-H, compare lane 3 with 1), which is consistent with the fact that there is enough H-NS in stationary phase wild type cells (Figure 3) to partially repress those virulence genes. Although the effect of *hns* on cell growth will be complex, an uncontrolled expression of the LEE genes and the T3SS is likely to be detrimental to the fitness of the cell [15]. Moreover, the expression level of EHEC virulence genes in the *hns*





**Figure 4** *SspA* is upstream of H-NS in the regulatory network of virulence gene expression in EHEC. The expression of virulence genes in wild type EHEC EDL933 (lane 1), *sspA* (lane 2), *hns* (lane 3) and *hns sspA* (lane 4) mutant derivatives was determined by primer extension analyses using labeled DNA oligos specific to the transcripts of *LEE1/ler* (A), *LEE2/espZ* (B), *LEE3/escV* (C), *LEE4/sepL* (D), *LEE5/tir* (E), *map* (F), *grlRA* (G) and *stcE* (H). In each reaction, the *ompA* transcript served as an internal control. Samples were prepared and analyzed as described in the legend of Figure 1. The relative transcript levels of target genes normalized to that of *ompA* are indicated by the numbers in parenthesis.

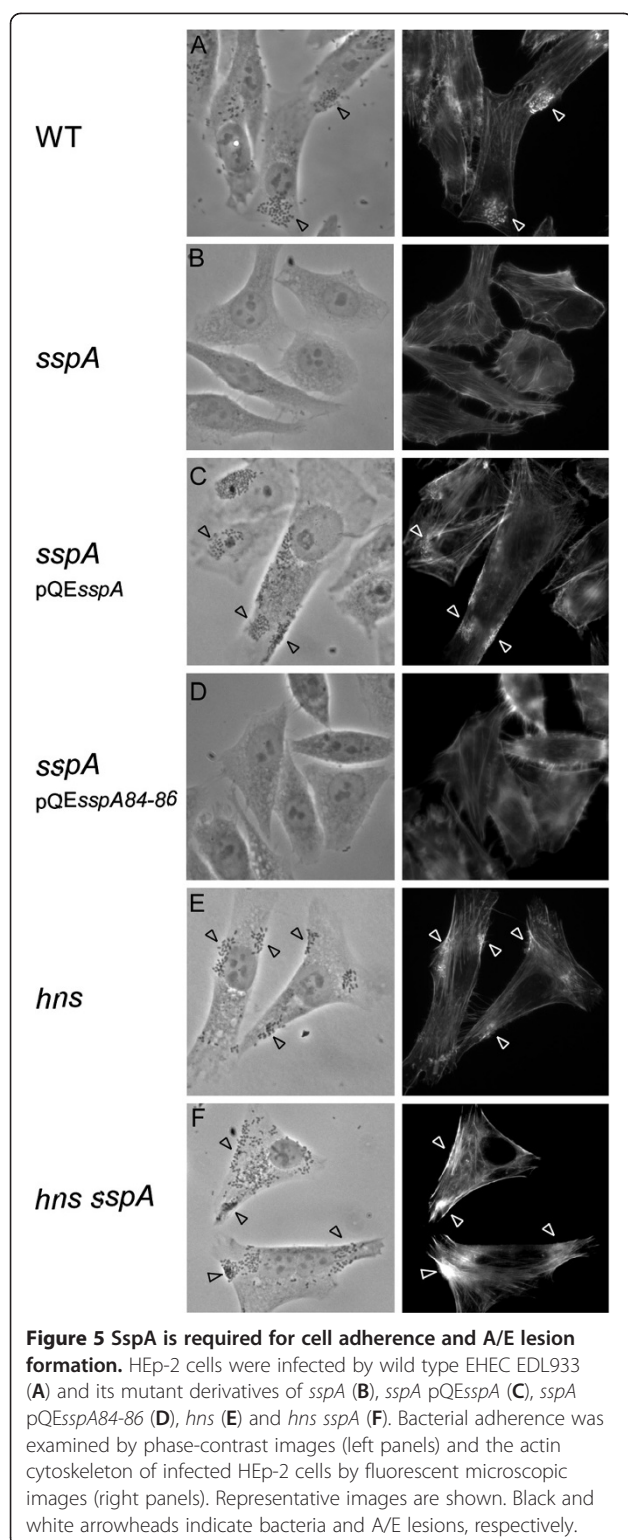
*sspA* double mutant was within the range of the level observed for the *hns* single mutant (Figure 4A-H, compare lane 4 with 3). Thus, our data strongly indicate that *SspA* is located upstream of H-NS in the regulatory cascade controlling the virulence gene expression in EHEC. However, *SspA* might also directly activate virulence gene expression in addition to controlling H-NS levels.

#### ***SspA* is required for cell adherence and A/E lesion formation**

Since the expression of LEE-encoded genes involved in A/E lesion formation was decreased in a *sspA* mutant and increased in a *hns sspA* double mutant (Figures 1 and 4), we predicted that *SspA* affects lesion formation in a H-NS-dependent manner. To address this, we infected HEp-2 cells with wild type, *sspA*, *hns* and *hns sspA* mutant derivatives of EDL933, and determined the ability of these strains to form A/E lesions *in vitro*. To this end we used the qualitative fluorescent actin staining (FAS) assay [53], where actin filaments are stained with FITC-phalloidin to detect A/E lesions that are visualized as condensed actin directly beneath adherent bacteria. Whereas infection with wild type EHEC was associated with the appearance of microcolonies of adherent bacteria and A/E lesion formation on 70% of the HEp-2 cells (Figure 5A), the *sspA* mutant was unable to adhere and form A/E lesions (Figure 5B) as determined from examination of more than 50 HEp-2 cells. The A/E lesion phenotype of the *sspA* mutant was restored when complementing with *sspA* *in trans* from pQE*sspA* (Figure 5C), whereas mutant *sspA* supplied from

pQE*sspA*84-86 (Figure 5D) did not complement pedestal formation of the *sspA* mutant, verifying that the surface-exposed pocket is functionally important for *SspA* to affect virulence of EHEC. Consistent with the finding that *SspA* regulates LEE expression through H-NS, the *sspA* mutant restored the ability to form A/E lesions in the absence of *hns* in the *hns sspA* background as in the *hns* single mutant (Figure 5E-F). However, the *hns sspA* double mutant seemed to form A/E lesions to a higher degree than the *hns* single mutant, which indicates that *SspA* also affects the expression of virulence genes involved in A/E lesion formation independently of the H-NS-mediated regulation. Moreover, the finding that the cell adherence ability of the *sspA* mutant was restored when deleting *hns* indicates that a factor negatively regulated by H-NS is required for the adherence of EHEC to epithelial cells. The long polar fimbria, LpfA, which is part of the H-NS/Ler regulon and is required for cell adherence of EHEC [32,54,55], might represent such a factor. Altogether, the cell adherence and A/E lesion phenotypes of the *sspA* mutant are consistent with the finding that *SspA* positively regulates the expression of genes encoding the T3SS including those of the LEE by negatively affecting H-NS levels.

The correlation between the effects of *sspA* on the transcription of H-NS/Ler-regulated virulence genes and on A/E lesion formation upon infection of HEp-2 cells supports the conclusion that *SspA* upregulates the expression of LEE and other virulence genes by reducing the accumulation of H-NS in the cell. A reduced cellular H-NS level mediated by *SspA* will derepress the H-NS



regulon and thereby allow the expression of transcriptional activators such as Ler and GrlA. These two activators then form a positive transcriptional regulatory loop partially by preventing H-NS-mediated repression [28]. Accumulation of Ler will in turn antagonize H-NS function

and with that enhance the expression of virulence genes controlled by Ler [26]. At present, the molecular mechanism behind SspA-mediated regulation of the H-NS level during stationary phase and in infection to facilitate virulence gene expression in EHEC is unknown. Also, it remains to be determined whether SspA directly affects transcription of virulence genes as is the case for SspA in *Francisella tularensis*, where SspA along with two other transcription factors and ppGpp activates transcription to link the nutritional status to virulence gene expression [56,57].

We observed that SspA positively affects additional H-NS-controlled virulence traits of EHEC such as stationary phase-induced acid tolerance (data not shown), which enables survival of the pathogen during passage through the low pH environment of the human gastrointestinal tract, and thereby contributes to a low infectious dose [58,59]. Also, *sspA* positively affects EHEC motility (data not shown), which could influence virulence as motility enables the pathogen to penetrate the intestinal mucus layer during colonization of host cells. This further supports an important role of *sspA* in EHEC virulence. Further experiments studying wild type and *sspA* mutant derivatives of the A/E pathogen *Citrobacter rodentium* in a mouse model could help determine whether *sspA* is required for virulence *in vivo*.

## Conclusions

We established an important role of SspA in the regulation of LEE- and non-LEE-encoded virulence factors of a T3SS, which is important for A/E lesion formation by EHEC. SspA downregulates H-NS levels allowing the expression of EHEC virulence genes, which are part of the H-NS/Ler regulon. Virulence genes in many bacteria are horizontally acquired genetic elements and subject to repression by H-NS. Thus, our study indicates that SspA potentially plays an important role in the pathogenicity of many bacterial pathogens in general.

## Methods

### Standard procedures

Standard DNA techniques, agar plates and liquid media were used as described [60]. Restriction endonucleases, T4 DNA polynucleotide kinase- and ligase (New England Biolabs) and the Expand High Fidelity PCR System (Roche Applied Sciences) were used according to manufacturer's instructions. DNA sequencing was performed by the National Cancer Institute DNA Sequencing MiniCore facility. Bacteria were grown at 37°C in LB or DMEM (Invitrogen #11885) media supplemented with ampicillin (100 µg/ml), chloramphenicol (25 µg/ml) or kanamycin (25 µg/ml) as needed. HEp-2 cells (ATTC # CCL-23) were cultured in DMEM supplemented with 10% fetal bovine

serum (FBS), 100 U/ml penicillin and 100 µg/ml streptomycin at 37°C in 5% CO<sub>2</sub>.

### Strain and plasmid constructions

Oligonucleotides used in this study are listed in Table 1. Gene deletions were constructed in EHEC O157:H7 EDL933 strain ATCC 700927 (Perna et al. 2001) by Lambda Red-mediated recombination using linear DNA fragments as described [61]. An in-frame deletion of *sspA* was created as previously described [44] resulting in strain DJ6010 (ATCC 700927  $\Delta$ *sspA*). The DNA fragment used for making the *sspA* deletion was amplified by PCR from pKD13 with primers PKD13sspAUS2 and PKD13sspADS. An *hns* deletion mutant derivative of strain ATCC 700927 was made by inserting a chloramphenicol resistance-encoding *cat* cassette, which was PCR amplified from pKD3 [61] using primers  $\Delta$ *hns*92-1 and  $\Delta$ *hns*92-2, 276 nt from the *hns* translation initiation codon (strain DJ6011). An *sspA hns* double mutant (DJ6012) was constructed by introducing the  $\Delta$ *hns::cat* deletion into strain DJ6010. All gene deletion constructs were verified by PCR amplification using primer sets *sspABUS/sspABDS* and *hnsUS2/hnsDS2*. In addition, Western blot analysis using polyclonal antibodies specific to the respective proteins confirmed the *sspA* and *hns* mutant strains. Plasmid pACYCler (pDJ610) contains a ~ 800 bp DNA fragment encoding *ler* expressed from its two native promoters cloned into the *Hind*III/

*Bam*HI sites of pACYC184. The DNA fragment was PCR amplified from EDL933 genomic DNA using oligos *lerUS2/lerDS2*.

### RNA isolation

DMEM is known to enhance the expression of the T3SS, which was detrimental to growth of the *hns* mutant EHEC derivatives (data not shown) that already exhibit increased T3SS expression in the absence of H-NS-mediated repression. Therefore, virulence gene expression was monitored in cells grown in LB, where a mid-level expression of the T3SS occurs. Overnight cultures of wild type and mutant derivatives of EDL933 ATCC 700927 were diluted 1:1000 in LB, supplemented with antibiotics if necessary, and grown aerobically at 37°C to an optical density at 600 nm (OD<sub>600</sub>) of ~ 3.0 (stationary phase). Samples of the cultures corresponding to ~7.5 × 10<sup>9</sup> cells were collected and RNA was stabilized immediately by addition of RNeasy Protect Bacteria reagent according to manufacturer's protocol (QIAGEN). Total RNA was purified using MasterPure™ total RNA purification kit as recommended by the manufacturer (Epicentre). Contaminating DNA in the RNA preparations was removed by DNaseI treatment. Isolated RNA was quantified based on measurements of absorption at 260 nm. The quality of RNA was evaluated by determining the ratio of absorption at 260 nm and 280 nm, which was within the preferred range of 1.7 to 2.1, and by agarose gel electrophoresis.

**Table 1 Oligonucleotide primers used in this study**

Application	Name	Oligonucleotide sequence (5' to 3')
Strain construction	PKD13sspAUS2	ACTATCATCCAATTTCTGCCAAATGTCGGGTATTGCTCAGGAGGTTCTTTCATGATGTCGGGGATCCGTCGACCTGC
	PKD13sspADS	AGATTAACCTCGGCCAGACGCATTTCACGTTCTGCTTCAGTGAGGCTGGAGCTGCTTCGA
	sspABUS	GATTGGATCCGGCTTCGTTACTCGTGACGCT
	sspABDS	GATTGAAGCTTACTTCACAACGCGTAATGC
	<i>hns</i> Δ92-1	GAGCTGCTGAATAGCCTTGCCGCGTTAAATCTGGCACCAAAGCTAAACGTGCTTGTTAGGCTGGAGCTGCTTCG
	<i>hns</i> Δ92-2	GGTTTTAGTTTCGCCGTTTCGTCAACGTAGCTATATTTTGCCGGACGCTTATGAATATCCTCCTTAGTTC
	<i>hnsUS2</i>	CCATGGAATTCACCATGAGCGAAGCACTTAAATCTGAACA
	<i>hnsDS2</i>	CCAGGTCTGCAGTTATTGCTTGATCAGGAAATCG
Plasmid construction	<i>lerUS2</i>	GATTGGATCCGCTGCGACTGCGTTGCTTGCT
	<i>lerDS2</i>	GATTGAAGCTTCCAGCTCAGTTATCGTTAT
Primer extension	<i>lerPE</i>	GCTTCCTGCTGTAGA
	LEE2PE	GCTGCTTCATTGATCTTTCTCC
	LEE3PE	CAATTTCAACACGGTTATC
	LEE4PE	CAGATGCGGGTTTTGATTAAATTC
	LEE5PE	CATTGGGATTATGACCAAGA
	mapPE	GGACTAAACATGCTATAAACC
	griRPE	CCTTCCCCACAGGAGTCTTC
	stcEPE	GGCAAGGATCGTACATGA
	ompAPE	ACGAGATAACACGGTTAAATCC



### Primer extension analysis

Primer extension reactions were carried out on 8 µg of total RNA using the AMV reverse transcriptase primer extension system according to manufacturer's instructions (Promega). The <sup>32</sup>P-labeled DNA oligos (1 pmol) were used to detect the transcripts of interest: *ler*PE (*LEE1/ler*), *LEE2*PE (*LEE2/espZ*), *LEE3*PE (*LEE3/mpc*), *LEE4*PE (*LEE4/sepL*), *LEE5*PE (*LEE5/tir*), *map*PE (*map*), *grl*PE (*grlRA*) and *stc*PE (*stcE*). The <sup>32</sup>P-labeled DNA oligo *ompA*PE was used as an internal control in each extension reaction to detect the transcripts from *ompA* P1 and P2. The DNA oligos were 5'-end labeled with (γ-<sup>32</sup>P) ATP (GE Healthcare) using T4 polynucleotide kinase (New England Biolabs). The cDNA products were separated on a 6 % denaturing gel along with a labeled φX174 *hinf*I DNA size marker (Promega) and visualized by autoradiography. The lengths of the cDNA transcripts *ler*, *espZ*, *mpc*, *sepL*, *tir*, *map*, *grlRA*, *stcE*, *ompA* P1, and *ompA* P2 were around 250, 120, 147, 122, 142, 135, 162, 125, 89 and 93 nt, respectively. No DNA product was detected in the absence of RNA. Transcript levels were quantified using ImageJ software [62] and normalized to *ompA* transcript levels. The primer extension experiments were carried out at least twice and similar results were obtained.

### Western analysis

Total protein was prepared from cultures grown in LB at 37°C to OD<sub>600</sub> ~ 3.0. Samples containing equal amounts of total protein equivalent to 0.03 OD<sub>600</sub> units of cell culture were prepared and analyzed essentially as previously described [44]. Polyclonal antibodies against H-NS or Fis were used to detect the respective proteins. The western blots were developed using ECL plus reagents (GE Healthcare) and quantified with a FluorChem imaging system (Alpha Innotech). The western analysis was carried out at least twice, and similar results were obtained.

### Assay for the presence of A/E lesions on HEp-2 cells

The ability of EHEC EDL933 (ATCC 700927) wild type and its mutant derivatives to adhere and form A/E lesions on HEp-2 cell monolayers was evaluated using the fluorescent actin staining assay as described [53]. Bacterial cells were grown without aeration for 16–18 h at 37°C in tryptic soy broth that was supplemented with antibiotics if needed. Prior to infection cells were diluted 1:5 in infection medium (DMEM supplemented with 2% FBS and 0.5% mannose) and incubated at 37°C 5% CO<sub>2</sub> for 2 h. About 2 × 10<sup>6</sup> bacteria (M.O.I. ~ 10) in 100 µl were added to semi-confluent HEp-2 cell monolayers grown on glass coverslips in a 6-well plate (Multiwell™ Falcon #353046). After infection for 4–5 h, monolayers were fixed with 4% formamide in PBS, washed three times with PBS, permeabilized with 0.1% Triton X-100 in

PBS, and then stained with Alexa Fluor 488 phalloidin (Invitrogen). Coverslips were mounted on slides using Prolong Gold antifade reagent (Invitrogen) and the edges of the coverslip were sealed with cyto-seal-60 (Richard-Allan Scientific). The samples were visualized using a Zeiss Axiophot II microscope equipped with a 40X objective, epifluorescence filters and a 1.25 optovar (Carl Zeiss MicroImaging Inc.). Images were captured with a charge-coupled device camera (Micromax) using IPL lab software. For each bacterial strain the assay was carried out independently at least three times and at least 50 HEp-2 cells were visually examined.

### Competing interests

The authors declare that they have no competing interests.

### Authors' contributions

Experiments were designed by AH and DJ. Experiments were performed by AH. The manuscript was written by AH and DJ. Both authors have read and approved the final manuscript.

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